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The Intracellular Distribution of Solanesol and Plastoquinone in Green Leaves of the Tobacco Plant

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Plastoquinone (koflerquinone) (I) has been isolated in relatively large quantities from the green leaves of a number of different plants (Crane,

1959a; Crane & Lester, 1958; Kofler, 1946; Kofler *et al.* 1959). Further, the quinone has been shown to be associated with the chloroplasts of green

leaves (Bishop, 1958; Crane, 1959b), and in preparations of this cell fraction it undergoes light-induced reduction to the quinol (Bishop, 1959, 1961; Crane, 1961). A more detailed study of this last process has indicated that the quinone plays an important part in light-catalysed electron transport and possibly in photophosphorylation (Arnon, 1961).

Ubiquinone (coenzyme Q) (II), on the other hand, is associated with the mitochondria of green leaves (Crane, 1959b). The function of ubiquinone has been studied in animal tissues, for these provide a much richer source of the compound. Here again more ubiquinone is found in the mitochondria than in any one of the other cell fractions (Aiyar & Sreenivasan, 1961, 1962a, b; Hemming, Pennock & Morton, 1958, and unpublished work; Seshadri-Sastry, Jayaraman & Ramasarma, 1961). It seems to function here as a component of the electron-transport chain (see, for examples, Green, 1961) and must be considered as a candidate for a part in oxidative phosphorylation (e.g., Clark & Todd, 1961; Ramasarma, 1961). Like the vitamin K₂ series of compounds, both of the above quinones have long isoprenoid side chains containing from six to ten isoprene residues (Gloor, Isler, Morton, Rüegg & Wiss, 1958; Lester, Crane & Hatefi, 1958). Only one plastoquinone has so far been fully characterized; this is the isoprenologue containing nine isoprene residues in its side chain. Crane (1961) has provided evidence for the existence in alfalfa, the fir and brown algae of a plastoquinone with a longer side chain, and Kegel, Henninger & Crane (1962) have also found one, and possibly two, isoprenologues in spinach chloroplasts.

Current ideas on the biosynthesis of the ubiquinones involve the condensation of a substituted benzoquinone with the corresponding polyisoprenoid alcohol. The latter is present in the form of the pyrophosphate. Thus Stoffel & Martius (1960) conclude that there is present in the liver of the rat an enzyme that catalyses the interaction of solanesyl pyrophosphate and 2,3-dimethoxy-6-methyl-1,4-benzoquinone to yield ubiquinone and phosphate. Small amounts of solanesol (III) have been found in the liver of the rat (Gloor & Wiss, 1960).

However, when radioactive mevalonic acid was administered to the rats, radioactivity was not incorporated into the solanesol, although it was recovered from the side chain of the liver ubiquinone-45 (UQ₅). Mevalonic acid has been shown

by Reid (1961) to be a precursor of solanesol in the tobacco plant. There is, therefore, some doubt as the nature of the precursor of the side chain of ubiquinone in the rat.

Several workers have shown that biosynthesis of ubiquinone almost certainly occurs in a number of animal tissues (Lawson, Threlfall, Glover & Morton, 1961; Stoffel & Martius, 1960). For this reason one might expect that those tissues that yield ubiquinone-50 will also yield the isoprenologue of solanesol that contains ten isoprene units, i.e. solanesol-50. Despite a thorough search, such a compound could not be detected in a number of animal tissues (Pennock, Hemming & Morton, 1960); a C₁₀₀ isoprenoid alcohol (dolichol) was isolated instead. Whether or not this is produced by the condensation of two molecules of solanesol-50 has not yet been established.

The yield of solanesol from certain plant tissues is much greater than that claimed for animal tissues. Solanesol was first isolated, and in relatively high yield (0.4% dry wt.), from tobacco leaves by Rowland, Latimer & Giles (1956), but its concentration in other green leaves appears to be considerably lower than this. However, the fact that it has been isolated from tissues rich in plastoquinone-45 implies that it might well be the precursor of the side chain of the latter.

In most of the plants studied the ubiquinone has ten isoprene residues in its side chain. The concentration of this quinone is usually low (Crane, 1959a, b; J. Stevenson, unpublished work), and as yet the corresponding solanesol-50 has not been isolated from green leaves. On the other hand, the spadix of *Arum maculatum* is very rich in ubiquinone-50 (32 mg./kg.) and solanesol-50 has recently been isolated from this tissue. Dolichol was also found but no solanesol-45. The yield of plastoquinone-45 was low (0.7 mg./kg.) (Pennock, Hemming & Morton, 1962, 1963). Such chemical studies on plant tissues therefore indicate that the side chains of the quinones might be derived from the corresponding isoprenoid alcohols, possibly in a way similar to the scheme proposed by Martius for animal tissues.

In order to pursue these problems we have studied the intracellular distribution of solanesol and plastoquinone in tobacco leaves.

EXPERIMENTAL

Isolation of cell fractions. Fresh washed leaves of tobacco, *Nicotiana tabacum virginiana*, were collected in the middle of September and were dried by pressing between towels. The main veins of the leaves were removed and the remaining material (900 g.) was cut into small pieces with scissors. This tissue was then treated in a manner similar to that described by Mayer & Friend (1960), except that tris-HCl

was replaced by phosphate buffer. It was macerated in a medium (pH 7.8) made up by mixing 438 g. of sucrose, 732 ml. of 0.2M- Na_2HPO_4 , 68 ml. of 0.2M- KH_2PO_4 and 80 ml. of 0.4M-KCl, and making the volume up to 3200 ml. with water. Charges of 100 g. of tissue and 200 ml. of medium were disrupted in a top-drive macerator (Townson and Mercer Ltd.) at full speed for 1 min. The leaf debris was removed from nine such suspensions by filtration through cheese-cloth and the filtrate was centrifuged at 230g for 1 min. The leaf debris was discarded and the supernatant fluid was then centrifuged at 950g for 10 min. The sedimented material was the chloroplast fraction. The supernatant fluid was then centrifuged at 9500g for 20 min. The resulting residue is designated the 'mitochondrial' fraction, and the final supernatant fluid the 'supernatant'. Most of the 'mitochondrial' fraction is of non-mitochondrial origin (see below). The entire process of maceration and centrifuging was carried out at 0-5°.

The chloroplast fraction was suspended in water, with a Potter-Elvehjem homogenizer, to give a total volume of 250 ml. In a similar way 250 ml. of 'mitochondrial' suspension was prepared.

Determination of chlorophyll. A small volume (0.5-1.5 ml.) of each suspension was pipetted into a stoppered tube and shaken with methanol. The mixture was centrifuged, the methanol was decanted off and the extraction repeated until all of the chlorophyll had been removed. The combined extracts from each fraction were made up to an exact volume in volumetric flasks and the extinctions of each solution were measured at 665 and 680 μ with a Unicam SP. 600 spectrophotometer.

The concentration of chlorophyll was calculated by using the following equation (see Vishniac, 1957):

$$\text{Total chlorophyll (mg./ml.)} = 0.0255E_{680\mu} + 0.004E_{665\mu}$$

Determination of protein. Small volumes of each suspension were treated according to the biuret method of Cleland & Slater (1953). A standard curve was prepared with fat-free casein (Genatosan). It was necessary to extract the plant protein precipitate several times with methanol to remove the chlorophyll. The determination was also carried out on a small sample of whole leaf.

Assay of plastoquinone and solanesol. To extract the lipids, the lipoproteins of the fractions were denatured by adding ethanol (4 vol./vol. of suspension) and heating. The ethanol concentration was decreased by adding water (20 vol./original vol. of suspension) and the mixture was then extracted three times with ether. The combined ethereal extracts were dried over anhydrous sodium sulphate and the solvent was then removed. The lipids were dissolved in light petroleum (b.p. 40-60°) and chromatographed on acid-washed Brockmann grade III alumina. Plastoquinone was eluted in an early fraction [2% (v/v) ether in light petroleum] and solanesol later [mainly by 8% (v/v) ether in light petroleum]. The infrared- and ultraviolet-absorption spectra of each fraction were recorded.

Fractions containing plastoquinone exhibited maximal absorption at 254 μ in ethanol. This peak disappeared on adding sodium borohydride and was replaced by a weaker one at 290 μ . The plastoquinone was assayed by this change in absorption, a value of $\Delta E_{1\text{cm}}^{1\%}$ at 254 μ of 200 for the pure compound being used.

Small samples of chromatographic fractions likely to contain solanesol were spotted on thin layers of silica gel G

(Merck). The presence or absence of solanesol was confirmed by using marker spots of authentic solanesol-45 and staining the plates with iodine after development with 1% (v/v) methanol in benzene.

Those fractions containing a reasonably high concentration [greater than 50% (w/w)] of solanesol were chromatographed (as lines made up of series of spots) directly on thin plates of silica gel. After development, a band of the gel corresponding in position to marker spots of solanesol-45 was scraped from the glass plate and extracted with ether. The nature of the extracted material was checked by infrared spectroscopy and each fraction was weighed. Those fractions resulting from the chromatography on alumina that contained lower concentrations of solanesol were acetylated, and the acetylated material was chromatographed on acid-washed Brockmann grade III alumina. Solanesyl acetate was eluted by 1% (v/v) ether in light petroleum (b.p. 40-60°). A less polar acetate was found in the earlier petroleum fraction and a small amount of more polar material was eluted from the column later. Occasionally the petroleum fraction contained a little solanesyl acetate. The combined fractions containing solanesyl acetate were saponified, and the unsaponifiable material was extracted with ether and chromatographed on thin layers of silica gel as described above.

Rowland & Latimer (1959) have reported that some of the solanesol in the tobacco leaf is esterified with fatty acids. To check this possibility a portion of whole-leaf lipid was chromatographed on alumina, and the early fractions, containing esters, were saponified and then investigated for the presence of solanesol as described above. Solanesol was not detected and it was therefore assumed that most of the solanesol in the leaves was not esterified.

Reversed-phase partition chromatography on paraffin-impregnated paper confirmed that the plastoquinone and the solanesol contained nine isoprenoid residues. Thus plastoquinone had R_F 0.24, the same as authentic plastoquinone-45, when dimethylformamide was used as the mobile phase. Solanesol had R_F 0.80 when the mobile phase was acetone-water (9:1, v/v). This value is the same as that of authentic solanesol-45 and is distinct from that of solanesol-50 (R_F 0.71).

Infrared absorption of solanesol. The infrared-absorption spectrum of solanesol-45 is recorded in Fig. 1 (see also Rowland *et al.* 1956). The important bands selected for scrutiny were the O-H stretching band at 2.9 μ in conjunction with the C-O stretching band at 10 μ , and the isoprenoid band at 11.92 μ in conjunction with the C-C stretching of unconjugated double bonds at 6.0 μ .

Fig. 1. Infrared-absorption spectrum of solanesol-45.

RESULTS AND DISCUSSION

The yields of chlorophyll and protein from the cell fractions and whole leaf were calculated for that expected from 900 g. of leaf. They are recorded in Table 1.

It was calculated from these values that 30.4 % of the chlorophyll of the whole leaf was recovered in the cell fractions. The corresponding recovery of protein is 29.7 %. It seems then that the cell fractions were derived from about 270 g. of whole tissue and that the remaining 630 g. was discarded as leaf debris.

It is also possible to estimate the degree of contamination of the mitochondrial and supernatant fractions by chloroplast fragments. In the chloro-

plast fraction 100 mg. of chlorophyll corresponds to 1.37 g. of protein. Hence 1.32 g. (71.4 %) of the 'mitochondrial' protein is probably derived from the chloroplasts. Similarly the 'supernatant'-fraction protein probably contains 0.525 g. (7 %) of protein of chloroplast origin. The recovery of plastoquinone and solanesol from the cell fractions is recorded in Table 2. The amounts of solanesol and plastoquinone respectively found in the mitochondria were 22.5 and 19.7 % of those found in the chloroplasts. The corresponding value for chlorophyll was 22.1 %. It thus seems that the solanesol and plastoquinone found in the mitochondrial fraction originate from contaminating chloroplast fragments. This is also obvious from Table 3. The concentrations of solanesol and plastoquinone/g. of chlorophyll are, respectively, approximately the same in the chloroplasts and mitochondria. Table 3 also shows that approximately 55 % (240 μ moles/g. of chlorophyll) of the plastoquinone and 70 % (105 μ moles/g. of chlorophyll) of the solanesol recovered in the supernatant fraction was not associated with chlorophyll, and therefore probably not derived by disruption of chloroplasts. It is possible that they were both derived from small undeveloped plastids.

The recovery of plastoquinone, solanesol and protein from the three cell fractions has been corrected for contamination by particles derived from the chloroplasts (Table 4). In making this correction it has been assumed that chlorophyll occurs only in the chloroplasts and that the concentrations of these three constituents/mg. of chlorophyll are the same in whole and disrupted chloroplasts. Table 4 shows that solanesol-45 accompanies plastoquinone-45 in the chloroplasts and, to a slight extent, in the supernatant fraction. It is not

Table 1. *Yield of chlorophyll and protein from 900 g. of whole leaf*

Experimental details are given in the text.

Fraction	Chlorophyll (mg.)	Protein (g.)
Chloroplasts	438.0	6.00
'Mitochondrial'	96.6	1.85
Supernatant	38.4	7.66
Whole leaf	1818.0	52.91

Table 2. *Yield of plastoquinone and solanesol from the tobacco-leaf cell fractions obtained from 270 g. of leaf tissue*

Experimental details are given in the text.

Fraction	Plastoquinone (μ moles)	Solaneseol (μ moles)
Chloroplasts	89.2	17.8
'Mitochondrial'	17.6	4.0
Supernatant	17.0	5.6

Table 3. *Concentration of plastoquinone and solanesol in the cell fractions*

Experimental details are given in the text.

Fraction	Concn. (μ moles/g. of chlorophyll)		Concn. (μ moles/g. of protein)	
	Plastoquinone	Solaneseol	Plastoquinone	Solaneseol
Chloroplasts	203.6	40.6	14.8	2.17
'Mitochondrial'	182.2	41.4	9.5	2.16
Supernatant	442.7	145.8	2.2	0.73

Table 4. *'Corrected' yield of plastoquinone, solanesol and protein from the cell fractions of 1 kg. (wet wt.) of tobacco-leaf tissue*

Experimental details are given in the text.

Fraction	Plastoquinone		Solaneseol		Protein (g.)
	(μ moles)	(mg.)	(μ moles)	(mg.)	
Chloroplasts	424	317	86	54	29.04
'Mitochondrial'	0	0	0.4	0.25	1.96
Supernatant	34	25	15	9.0	26.44

yet decided whether in fact the isoprenoid alcohol can be formed from mevalonic acid in the chloroplasts and also whether this alcohol (possibly in the form of a pyrophosphate) can then condense with a quinone to form plastoquinone. In animal tissues the corresponding condensation to form ubiquinone occurs in the mitochondria (Stoffel & Martius, 1960).

SUMMARY

1. Three fractions were isolated from fresh leaves of tobacco: (a) one containing chloroplasts, (b) one containing mitochondria and broken chloroplasts, and (c) a supernatant fraction.

2. Both plastoquinone (424 μ moles/kg. of leaf tissue) and solanesol (86 μ moles/kg. of leaf tissue) were concentrated in the chloroplasts.

3. Both compounds were the homologues containing nine isoprenoid units, i.e. solanesol-45 ($C_{45}H_{73}OH$) and plastoquinone-45 each with a C_{45} side chain.

4. All the isolated solanesol was unesterified.

5. Some plastoquinone (34 μ moles/kg. of fresh leaves) and solanesol (15 μ moles/kg. of fresh leaves) found in the supernatant fraction were not associated with the chloroplasts.

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The Occurrence of a Long-Chain Ether Analogue of Phosphatidylethanolamine in Brain Tissue

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It has long been known that brain and some other animal tissues contain phospholipids whose phosphorus is not rendered water-soluble by mild alkaline and acid hydrolysis and which are not

sphingomyelins (Brant, 1949; Dawson, 1954; Edgar & Smits, 1959). One of these components has recently been shown to be an artifact, a cyclic acetal of glycerylphosphorylethanolamine pro-